Supplementation of Lactobacillus reuteri isolated from red jungle fowl along with mannanoligosaccaride improves growth performance, immune response and gut health in broiler Chicken

Sudhir Kumar JaiswaL
Central Avian Research Institute
Simi Tomar
Central Avian Research Institute
Vishesh Saxena
Central Avian Research Institute
Balraj S
Central Avian Research Institute
Sandeep Uniyal (sandeepuniyalvet18@gmail.com)
Guru Angad Dev Veterinary and Animal Sciences University

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Abstract

The present study was undertaken to investigate the effect of supplementation of *Lactobacillus reuteri* isolated from the intestine of red jungle fowl along with mannanoligosaccride (MOS) on growth performance, intestinal microbial count, immunity and expression of growth and immune related genes in broiler chicken. *Lactobacillus reuteri* was isolated from in the GIT tract of red jungle fowl and was utilized for growth bioassay in broiler. For this total 360 CARIBRO-Vishal broiler chicks were weighed individually and randomly allocated to nine treatment groups, each having five replicates with eight chicks in each following complete randomized block design (CRD). The experiment was conducted for 6 weeks duration. The nine treatment groups were control fed basal diet (T1), basal diet + Antibiotic growth promoter, bacitracin methylene disalicylate (BMD) @20mg/kg feed (T2), basal diet + commercial probiotic @ 0.1 g/kg feed (T3), basal diet + Lab isolated *Lactobacillus reuteri* @ $1x10^6$ CFU/g of fermented feed (T4), basal diet + Lab isolated *Lactobacillus reuteri* @ $1x10^7$ CFU/g of fermented feed (T5), basal diet + Lab isolated *Lactobacillus reuteri* @ $1x10^8$ CFU/g of fermented feed (T6), basal diet + Lab isolated *Lactobacillus reuteri* @ $1x10^6$ CFU/g of fermented + 0.1% MOS (T7), basal diet + Lab isolated *Lactobacillus reuteri* @ $1x10^7$ CFU/g of fermented + 0.1% MOS (T8), basal diet + Lab isolated *Lactobacillus reuteri* @ $1x10^8$ CFU/g of fermented feed + 0.1% MOS (T9). 20% of daily basal ration for broiler chicken was autoclaved and inoculated with 15% of Lactobacillus isolate broth culture having a viable count of 10^6, 10^7, and 10^8 CFU/ml and fermented at 37°C for 24 h before adding to daily ration afresh and was mixed well. Results of the present study revealed T9 group supplemented with Lab isolated *Lactobaillus reuteri* at the dose of $1x10^8$ CFU/g along with 0.1% MOS significantly ($P < 0.05$) improves body weight, body weight gain, immune response both humoral and cell mediated without effecting the feed intake and feed conversion ratio (FCR). Also the pathogenic bacteria count (Salmonella and E.coli) was significantly ($P < 0.05$) lower in the GIT of T9 group as compared to other groups. The significantly ($p < 0.05$) higher relative expression of growth related genes, IGF-1 and IGF-1R and immune related gene, IL-6 whereas IL-10 and TLR-4 expression were significantly ($P < 0.05$) down regulated in T9 group (Lab isolated *Lactobacillus reuteri* @ $1x10^8$ CFU/g of fermented feed + 0.1% MOS). So, it can be concluded from the present study that lactobacillus *reuteri* isolated for the GIT of the red jungle fowl along with MOS is effective in improving the growth performance, immune response and gut health of commercial CARIBRO-Vishal broiler chicken.

Introduction

The chicken gastrointestinal tract (GIT) is rich in microbial biodiversity of more then 500 phylotypes or over 1 million bacterial genes, which equates to 40–50 times of number in the chicken nuclear genome (Shang et al., 2018). This microflora has a role in nutrition, detoxification of certain compounds, growth performance, and protection against colonization of pathogens and influences health and well-being of host animals (Paul et al., 2022). When these live microorganisms administered in adequate amounts confer a health benefit on the host and with this, the concept of probiotic has been evolved (Al-Khalaifah, 2018). Lilly and Stillwell coined the term ‘probiotics’ in 1965, derived from Greek word “pro bios”, meaning “for life”. Probiotics as per World Health Organization (WHO) and Food and Agriculture Organization (FAO) are “live microorganisms, which when administered in adequate amounts confer a health benefit on the host”.

Probiotics have numerous advantages as they improves digestibility and utilization of nutrients, increases growth rate and productivity, inhibits disease producing organisms, prevents diarrhoea due to bacterial infections, reduces stress after vaccination, antibiotic therapy and transportation and stimulates the immune responses (Alayande et al., 2020; Jha et al., 2020). Antibiotic growth promoter (AGP) have been used at sub-therapeutic doses in poultry diets to prevent diseases and to promote growth performance for many decades but since the ban imposed by European union in 2006 on the use of these AGP in farm animals due to environmental and public health risk associated with emergences of antibiotic resistance (Castanon, 2007). Therefore, alternatives to the use of AGP must be found to promote growth or production at or near the genetic potential of the modern day poultry. In recent year's use of probiotics that enrich certain bacterial population in the digestive system are considered as alternatives to antibiotic growth promoters in poultry nutrition (Rivera-Pérez et al., 2021). Domestic birds raised under commercial conditions are vulnerable to a number of pathogens (Paul et al., 2021). The World Health Organization (WHO) has now urged egg and meat producers to use environment friendly alternative methods to control diseases. Phasing out of antibiotic growth promoters from poultry diets in Europe and recent moves toward reduction or removal of these compounds in other parts of the world will likely change the microbial profile of the GIT environment in commercial poultry. Hence every effort would be made to improve the gut efficiency through natural microflora and fauna for better nutrient utilization. Assuming that the Red Jungle Fowl being raised in natural habitats could represent the most natural GIT environment and may harbouring certain uncharacterized strains of microbes imparting better immunity and adaptability to these birds. It is the primary progenitor of the domestic chicken and native to southern Asia, particularly the jungles of India. Keeping above facts in view, the present study has been designed to effect of supplementation of *Lactobacillus reuteri* isolated from the intestine of red jungle fowl along with mannanoligosaccride (MOS) on growth performance, intestinal microbial count, immunity and expression of growth and immune related genes in broiler chicken raised under intensive system for high economic return.

Material And Methods

Isolation of Lactobacillus

Lactobacillus reuteri was isolated from gut sample of four Red Jungle fowl housed under uniform management and feeding conditions. The purified cultures were stored as 50% glycerol stock mixture (250 µl of broth culture in 1.75 ml of 50% glycerol+50% MRS broth medium) at -80°C until further use (Garriga et al., 1998). The isolates were sub-cultured at least 2 times before all of the assays. 1 ml from each isolate culture was used for CFU enumeration. Titration was done by serial dilution, plating and counting on MRS agar plates. After that the aliquots was adjusted to 10^6, 10^7 and 10^8 CFU/ml by using sterile PBS. Broiler (starter and finisher) ration used for feeding the birds in present study were fermented with *Lactobacillus reuteri* for this 20% of daily ration was autoclaved and daily inoculated with 15% of *Lactobacillus reuteri* broth culture having viable count of 10^6, 10^7 and 10^8 CFU/ml and fermented at 37°C for 24 hours before added to daily ration afresh and mixed well.

Experimental birds and housing management

360 day old CARIBRO-Vishal broiler birds were procured from the ICAR-CARI, Izatnagar hatchery. Each bird was weighed on arrival and randomly assigned to nine groups using completely randomized design (CRD). Each dietary treatment had five replicates having eight broiler birds in each replicate. All the experimental groups of birds are reared in the battery brooder fitted with waterer and feeder from day one to 42^{nd} day of age. Twenty-three-hour light was provided throughout the experimental period. Fresh and clean water were offered *ad labitum* throughout the experimental period. Proper ventilation was maintained in the shed. Daily feed intake, weekly body weight and Mortality, if any was recorded throughout the experimental feeding. The birds are vaccinated for common diseases following standard vaccination schedule.

Experimental feeding of birds

All the experimental birds fed basal diet composed of maize, soybean meal, de-oiled rice bran and fish meal was formulated to target the requirement of the essential nutrients for broiler chickens as per ICAR, 2013. T1 fed basal diet (BD), T2 BD+ Antibiotic BMD (Bacitracin Methylene Di-salicylate) @ 20mg/kg diet, T3 BD+ Commercial multi-strain probiotic (0.1g/kg feed) T4 BD+ Lab isolated *Lactobacillus reuteri* ($1x10^6$ CFU/g fermented feed) T5 BD+ Lab isolated *Lactobacillus reuteri* ($1x10^7$ CFU/g fermented feed) T6 BD+ Lab isolated *Lactobacillus reuteri* ($1x10^8$ CFU/g fermented feed) T7 BD+ Lab isolated *Lactobacillus reuteri* ($1x10^6$ CFU/g fermented feed)+MOS (0.1g /kg feed) T8 BD+ Lab isolated *Lactobacillus reuteri* ($1x10^7$ CFU/g fermented feed)+MOS (0.1g /kg feed) T9 BD+ Lab isolated *Lactobacillus reuteri* ($1x10^8$ CFU/g fermented feed)+MOS (0.1g /kg feed). Weighed amount of each test diet used during the starting period (0-3wks) and finishing period (4-6wks) were offered daily in five replicates of eight chicks each to ensure *ad libitum* feeding at all the time, but with care to avoid spillage and wastage of feed.

Growth performance and feed intake

Body weights of the birds were recorded at 0^{th} day, 1^{st}, 2^{nd}, 3^{rd}, 4^{th} , 5^{th} and 6^{th} week in the morning before offering the feed to birds. Body weights were taken by using platform digital balance. To measure weekly feed intake of the birds weighed quantity of respective diet was offered *ad libitum* daily to quadruplicate groups of each dietary regimen in the morning and the residue was weighed at the end of every week.

Immune response

Immune responses of the experimental birds as affected by different dietary dose of lab isolated *Lactobacillus reuteri* treatments were evaluated in terms of humoral and cell mediated immune response.

Humoral immune response

Humoral immunity was evaluated as antibody titre (HA) against 1% sheep red blood cells (SRBC) suspension. The microtitre haaemagglutinin (HA) procedure [10] was followed to measure total HA antibody titre in chickens on 5th and 10th day of post inoculation

In-Vivo Cell mediated immune response (CMI)

The CMI response to phytohaemagglutinin-P (PHA-P) mitogen was evaluated by the method of Cheng & Lamont (1988). PHA-P (1mg/ml of PBS) was injected intra-dermally in the left foot web of 8 birds/groups. Right foot web of the same birds received 0.1 ml sterile PBS and thus served as control. The skin thickness of foot webs (Right and Left) of injected birds of each group was measured by a micrometer at 0 and 24 hours after injection of mitogen. The foot web swelling was calculated by subtracting the skin thickness at 24 hours from that of 0 hours of injection of both foots. Foot web index (FWI) was calculated by subtracting the difference in thickness at 0 and 24 hrs of mitogen injected foot web with the difference in thickness of control foot web at 0 and 24 hrs.

GI tract microflora population

GIT contents were collected separately from crop, ileum and caecum at the end of experiment from broilers for bacterial enumeration viz Lactobacilli, Salmonella and E. coli. GIT contents were diluted 10-fold with buffered peptone water and vortexed for 2 min; 100 microliters of supernatant was smeared onto appropriate selective media in duplicate plates and incubated under optimum time-temperature combinations. After incubation period bacterial counts were recorded as CFU/ml.

Gene expression analysis

Growth and immunity related gene

At 6th week of age eight birds per group were sacrificed for collection of pectoral muscle and Ileum tissue sample. Total RNA was isolated from the tissue sample by standard TRIZOL method. The purity of RNA was checked before the preparation of first- strand cDNA. Prepared cDNA was stored at -20 ^{0} C and used for gene expression studies. Expression of IL-6, IL-10, TLR-4, IGF-1 and IGF-1R gene was quantified with using specific primer pairs (Table 1) for gene of interest (GOI) in Real-Time PCR. GAPDH was used as a reference gene

Statistical analysis

The data generated in the above experiment were statistically analyzed using IBM SPSS version 20 computer package. For comparison of groups, generalized linear model ANOVA procedure and Duncan's multiple range tests were used (Snedecor & Cochran 1994). The fold expression of gene was calculated by using the $2^{-\Delta\Delta Ct}$ method and analyzed by one way ANOVA

Results

Growth performance

The data pertaining to growth performance of experimental birds as influenced by various dietary treatments has been presented in Table 2. Body weight at 3rd week of age was observe as numerically higher in all dietary treatments groups as compared to control group but highest body weight was recorded

in T9 (1x10^8 CFU *Lactobacillus reuteri* + 0.1% MOS) group. The cumulative body weight at 6 week of age was significantly (P<0.05) higher in T9 followed by statistically similar T8, T2, T7, T6 and T3 whereas significantly lower body weight was recorded in T1. No significant difference (P>0.05) was observed in average body weight gain among the nine different dietary treatment groups in 0-3 and 4-6 week of age. The body weight gain (0-6 week) was significantly (P<0.05) higher in T9 followed by statistically similar T8, T7, T6, T3 and T2 whereas significantly (P<0.05) lower body weight was recorded in T1 followed by statistically similar T8, T7, T6, T3 and T2 group.

Feed Intake and Feed conversion ratio (FCR)

The data pertaining to feed intake (g) and FCR is presented in Table 3. The results indicate that no significant (P>0.05) difference was observed in feed intake among the different dietary supplemented groups during 0-3, 4-6 and 0-6 week of age. The result of FCR showed that there were no significant (P>0.05) differences between the different dietary groups and control group at 0-3, 4-6 and 0-6 week of age. The T9 group (1x10^8 CFU *Lactobacillus reuteri* + 0.1% MOS) showed numerically better FCR than the other groups whereas, the control group showed highest feed conversion ratio than others dietary treatment groups at all times

Intestinal microbial count

The least squares analysis of variance for $\log_{10}$ CFU/g of Lactobacillus, Salmonella and E. coli in crop, ileum and caecum of Broiler fed with different treatments is presented in Table 4. . Enumeration of Lactobacillus spp. Salmonella spp. and E.coli was conducted at 42th day of feeding trial in Broiler. The Lactobacillus count in crop was significantly (P<0.05) higher in T9 (1x10^8 CFU *Lactobacillus reuteri* + 0.1% MOS) group and significantly lower in T1 group whereas other treatments resulted in intermediate Lactobacillus count in crop at 6th week of age. The Lactobacillus count in ileum was significantly (P<0.05) higher in T9 followed by statistically similar T8 and lower in T1 and T2 whereas other treatments resulted in intermediate Lactobacillus count in ileum at 6th week of age. In caecum also T9 showed higher (P<0.05) Lactobacillus count than control and other dietary treatment groups.

Significantly (P<0.05) lower Salmonella count was observed in the crop and ileum of the broilers in T9 group as compared to all the treatment groups at 6th week of age. Salmonella count in caecum was significantly (P<0.05) lower in T9 followed by statistically similar in T8, T7, and highest Salomnella count was observed in T1 group at 6th week of age. The E.coli count in crop was significantly (P<0.05) lower in T9 group followed by statistically similar in T8, T3 and T6 groups whereas significantly (P<0.05) higher in T1 followed by statistically similar T7, T8, T3, T4, T5, T6 and T8 at 6 week of age. In ileum E. coli count was significantly (P<0.05) lower in all dietary treatments except T4 and T1. Similar finding was also observed in caecum with significantly (P<0.05) lower E. coli count in T9 group followed by statistically similar T8, T7, T6, T5, T4, T3 and higher count observed in T1 group. The results obtained in this work revealed that 1x10^8 CFU of *Lactobacillus reuteri* along with 0.1% MOS significantly increase lactobacillus count and reduce Salmonella and E. coli count in crop, ileum, and caecum.

Immune response

The results of immune response (humoral and cell mediated immune response) as affected by different treatments are presented in Table 5. The antibody titre (humoral immnutity) was significantly (P<0.05) lower in T1 group followed by statistically similar T4 and T5, whereas significantly (P<0.05) higher antibody titre was obtained in T9 group which did not differ significantly from treatment T8, T2, T7, T3, and T6 groups. The antibody titre, it is revealed that dietary inclusion of lab isolated Lactobacillus along with MOS significantly (P<0.05) enhanced the antibody titre values of broilers, more prominently in group T9 and T8. The cell mediated immunity was significantly higher in T9 group followed by statistically similar T8 group, whereas significantly (P<0.05) lower cell mediated immunity was obtained in control (T1) group which did not differ significantly from treatment T4, T3, T5, T2, T6 and T7 group.

Gene expression

Growth related gene

The results pertaining to the gene expression of IGF-1 and IGF-1R are illustrated in Fig 1 and 2.The mRNA levels of IGF-1 and IGF-1R gene were quantified by real-time PCR in breast muscle tissue at the end of 6th week. The relative fold expression of IGF-1 gene were significantly (P<0.05) up regulated in all dietary treatment groups compare to control (T1). The greater up regulation was observed in T9 group followed by statistically similar T8 and T2 group. Similar pattern was also observed in relative expression of IGF-1R gene, where higher relative expression was recorded in T9 and T2 group compared to other dietary treatment and control.

Immune related gene

The results of the gene expression analysis of IL-6, IL-10 and TLR-4 are illustrated in Fig 3, 4 and 5. The mRNA levels of IL-6, IL-10 and TLR-4 genes were quantified by real-time PCR in ileum tissue at the end of 6th week The significantly (P<0.05) lower relative fold expression of IL-6 gene was observed in treatment group T9 followed by statistically similar T2 and T8 group, whereas higher expression was observed in treatment group T1 followed by statistically similar T4 and T5 groups. The other dietary treatments yielded intermediate fold expression of IL-6 in broiler chicken. The relative fold expression of IL-10 gene were significantly (P<0.05) up regulated in T9 group followed by statistically similar T8, T7, T6, T3 and T2 groups whereas lower expression was observed in treatment control (T1) and other dietary treatments yielded intermediate fold expression of IL-10 in broiler chicken. The significantly (P<0.05) lower relative fold expression of TLR-4 gene was observed in treatment group T9 followed by statistically similar T2 and T3 group, whereas higher expression was observed in treatment group T1 and other dietary treatments yielded intermediate fold expression of TLR-4 in broiler chicken.

Discussion

Growth performance

The beneficial effect of any supplement is primarily judged through examination of the response in terms of growth of birds for which it is intended to be offered as a part of the diet. Accordingly, growth response of broiler chicken to dietary supplement was an important part of the study. The response criteria of feeding trial like growth performance viz., body weight, body weight gain, feed intake, feed conversion ratio. From this study it can be concluded that dietary supplementation of lab isolated *Lactobacillus reuteri* @ $1x10^8$ CFU along with MOS (0.1%) increases body weight and body weight gain at 6th week of age with no effect on feed intake and feed conversion ratio. This finding is in agreement with several reports demonstrating that probiotic supplemented to the birds improve the body weight, body weight gain and feed conversion ratio (Ramlucken et al., 2020; Rehman wt al., 2021; Zhang Sun et al., 2022; Chen et al., 2022 and Qiu et al., 2022). However, contrary to our observation body weight, daily weight gain and FCR of broilers were not influenced by the dietary supplementation with probiotics (Ramarao et al., 2000; Ergün et al., 2004; Arslan 2004 and Gyawali et al., 2022). The improvement in the body weight, daily weight gain, feed consumption and feed conversion ratio in the present study may be due to the increase in the digestion and absorption of the nutrients by increasing the secretion and activity of the digestives enzymes processes and reduction of the population of pathogenic bacteria by decreasing the intestinal P^H due to presence of the *lactobacillus reuteri* bacteria and also maintain the balance between beneficial and harmful bacteria which is required for sound gut health and optimum growth performance (Yaqoob et al., 2022).

Intestinal microbial count

Feeding of lab isolated *lactobacillus reuteri* $1x10^8$ CFU/g along with MOS (0.1%) to broiler chicken for 6 weeks significantly ($P<0.05$) increases Lactobacillus count in different parts of GIT (crop, ileum and caecum) with concomitant decrease in pathogenic organisms such as E. coli and Salmonella count. The results were in accordance with (Idoui et al., 2012; Vineetha et al., 2016 and Ashraf et al., 2009) who reported that reduction of E. coli and Salmonella count in intestine upon Lactobacillus feeding. The mechanism of the antimicrobial action may be due to the potential of Lactobacillus isolates to produce fatty acids, lactic acids, hydrogen peroxide diacetyl, acetoin and the small, heat-stable inhibitory peptides called bacteriocins (Soomro et al., 2004 and Simova et al., 2009) Other proposed mechanisms of pathogen inhibition by the probiotic micro-organisms include competition for nutrients, and by modifying the structure and function of the intestinal epithelium. Prebiotics such as MOS can also flush out pathogens from GIT serving as receptor sites for their adherence Vineetha et al., 2017)

Immune response

Probiotics as an alternative to antibiotics are considered to improve the health status and immunity in poultry birds. It has been reported that probiotic supplementation influenced the production of B-lymphocytes, T lymphocytes, anti-pro inflammatory cytokines and interleukins production in broiler chicken. In present study dietary supplementation of lab isolated *Lactobacillus reuteri* @ $1x10^8$ CFU along with MOS (0.1%) enhanced the immune response (humoral and cell mediated immune response) of broiler chicken compared to other levels of *Lactobacillus reuteri*. The results of the present study are in agreement with the finding of (Rehman wt al., 2021; Zhang Sun et al., 2022; Chen et al., 2022; Haghighi et al., 2006; Khaksefidi et al., 2006; Li et al., 2009; Stringfellow et al., 2011 and Mahmoud et al., 2013) who reported that

supplementation of Lactobacillus probiotic enhance immune response by greater lymphocytes cell proliferation, increased production of interleukins and lowering the production of pro-inflammatory cytokines.

Gene expression

Growth related gene

In this study we have found that higher relative expression of both IGF-and IGF-1R gene in birds fed $1x10^8$ CFU/g *Lactobacillus reuteri* along with 0.1 % MOS (T9 group) may be due to stimulation of glucose and amino acid uptake, protein synthesis and inhibition of protein degradation by lab isolated *Lactobacillus reuteri* (Duclos et al., 1993). On the similar lines Saleh et al., (2014) reported that expression of insulin like growth factor 1 (IGF- 1) and insulin like growth factor receptor 1 (IGF-1R) increased in *Aspergillus awamori*, FOS and combined *Aspergillus awamori* and FOS (symbiotic) fed group. The well documented biological role of IGF-1 is, but not limited to, increased glucose and amino acid uptake, and enhanced DNA and protein synthesis. The IGF I released into the circulation binds to its specific receptor called IGF-1 which stimulates cell proliferation (Okumura 1998). Guobin et al., (2011) noted that the IGF-1 is one of the main growth factors that stimulate protein synthesis in muscle tissue. Also, IGFs are important positive modulators of body and muscle growth in mammals and chickens. IGF-1 is a primary mediator of the effects of growth hormone (GH). Growth hormone, made in the anterior pituitary gland, is released into the blood stream and then stimulates the liver to produce IGF-1 which in turn stimulates systemic body growth, and has growth-promoting effects on almost every cell in the body, especially skeletal muscle, cartilage, bone, liver, kidney, nerves, skin, hematopoietic cell, and lungs. In addition to the insulin like effects, IGF-1 can also regulate cell growth and development, especially in nerve cells, as well as cellular DNA synthesis (Yakar et al., 2002). The IGF-I released into the circulation binds to its specific receptor called by IGF type1 receptor and finally stimulates cell proliferation (Okumura 1999). Thus, the better growth performance of birds in this study under $1x10^8$ CFU/g *Lactobacillus reuteri* along with 0.1 % MOS (T9) can be due to higher corresponding IGF-1 levels. This association of growth performance and IGF-1 expression was reported by many authors. Kita et al., (2002) stated that the changes in the plasma IGF-1 levels in the body alter the nutritional condition of young chicken and cause changes in body weight gain. Thus, from the current study the inclusion level of $1x10^8$ CFU/g *Lactobacillus reuteri* can be regarded as the requirement level of the chicken.

Immune related gene

In Chicken IL-6, secreted by T cells and macrophages, acts as a pro-inflammatory cytokine in association of the production of acute phase proteins. The IL-6 upregulation in chicken has been associated with Salmonella and Eimeria infection [Kaiser et al., 2006; Lynagh et al., 2002 and Wigley and Kaiser 2003). Therefore, a down regulation of IL-6 in the present study favours an anti-inflammatory response and shows that *Lactobacillus reuteri* had an anti-inflammatory effect in the gut. The results of present study are in agreement with the finding of (Chichlowski et al., 2007 and Lei et al., 2009) who reported the down regulation of IL-6 in the chicken gut when their diet was supplemented with probiotic. The probiotic bacteria could act in stabilizing intestinal inflammation by balancing the intestinal microflora, maintaining mucosal

barrier, modulating and improving the intestinal mucosal immune system, especially by keeping the balance of pro-inflammatory and anti-inflammatory cytokines and production of intestinal IgA (Isolauri et al., 2002 Hua et al., 2010 Ashraf and Shah 2104 and Zagato et al., 2014). The relative fold expression of IL-10 gene were significantly ($P<0.05$) up regulated in T9 Group (lab isolated lactobacillus1x10^8 CFU/g along with MOS 0.1 %). The IL-10 act as anti-inflammatory cytokine that controls the nature and extent of inflammatory responses to various microbial infections (Couper et al., 2008) and is involved in intestinal immunity and homeostasis (Manzanillo et al., 2015). Several reports have shown the regulation of IL-10 by probiotic bacteria (Christensen et al., 2002 and Chen et al., 2005). Similar to the results of the present study Chen et al., (2012) observed significant increase in IL-10 expression following the Lactobacillus supplementation in broiler chicken. However, in contrast to our study Haghighi et al., (2006) reported no significant changes in IL-10 expression in broiler chicken due to the Lactobacillus supplementation. The TLR-4 is the principal receptor for lipopolysacharide, which is a major component of the outer membrane of gram-negative bacteria (Kannaki et al., 2010). The decline in the expression of TLR-4 expression in T9 group in the present study means lesser receptor binding sites for the gram negative bacteria which results in the expulsion of pathogens like salmonella and E. coli from the gut. The down regulation of TLR-4 expression would be expected in the intestine of broilers fed the probiotic-supplemented diet because dietary inclusion of the probiotics decreased the population of gram-negative bacteria such as Coliform in the rectum of broilers (Lei et al., 2009).

Conclusion

In the present study supplementation of *Lactobacillus reuteri* improved growth performance (body weight gain), immune response (humoral and cell mediated immune response), reduces pathogenic gut bacteria (Salmonella and E. Coli) and upregulated expression of growth related genes (IGF-1 and IGF-1R) and immune related gene, IL-6 whereas expression of IL-10 and TLR-4 gene was down regulated in broiler. Thus, the Red jungle fowl specific lab isolate *Lactobacillus reuteri* may be used in commercial broiler production for enhancing growth and immunity.

Declarations

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Conflict of Interest Statement

The authors declare that they have no conflict of interest

Authors Contributions

All authors contributed equally to the work

Data availability

All the data is provided with the manuscript

Statement of Animal Ethics

The experiment was approved by Institutional Animal Ethics Committee and conducted under guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

Consent to participate

All authors give their consent to participate in the publication of this manuscript

Consent for publication

The authors give their consent for publication

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Tables

Table 1. Details of the primers used for real-time PCR analysis of gene in muscle and ileum tissue of broiler

S.No.	Gene	Primer	Annealing temperature (^{0}C)	Product size (bp)	Accession no.
1	GAPDH	F: 5'-CCGTCCTCTCTGGCAAAGTCC-3' R: 5'-AGCCCCAGCCTTCTCCATG-3'	**58**	**264**	NM204305
2	IL-6	F: 5' GAAATCCCTCCTCGCCAATCTGA -3' R: 5'TGAAACGGAACAACACTGCCATCT-3'	**58**	**219**	JN639847
3	IL-10	F: 5'-TGCGGGAGCTGAGGGTGAAGTTTG-3' R: 5'-CGCGGGGCTGGGCTGAGAG-3'	**52**	**215**	AJ621614
4	TLR-4	F: 5'-GTGCCACATCCATACAATAGAAGA-3' R: 5'-ATGGCCCAGATTCAGCTCCT-3'	56	256	JQ280467
5	IGF-1	F: 5' GGTGCTGAGCTGGTTGATGC-3' R: 5'CGTACAGAGCGTGCAGATTTAGGT-3'	**58**	**127**	JN942578
6	IGF-1R	F: 5'TAGATCCCTCTACCCTCCAA-3' R: 5'TCTGAAGATCCACTGAGGTACAG-3'	**57**	**291**	Heck *et al.*, 2003

Table 2: Body weight and Body weight gain in broilers fed on different dietary treatments.

Treatment	Body Weight (g)			Body Weight gain (g)		
	Day old	3 week	6 week	0-3 week	4-6 week	0-6 week
T1	39.85	533.55	1554.67^{a}	493.70	676.71	1514.82^{a}
T2	41.45	555.88	1631.86^{bc}	515.43	722.96	1591.41^{bc}
T3	40.00	543.35	1576.21^{abc}	503.36	689.34	1536.21^{abc}
T4	38.34	539.59	1561.25^{ab}	499.2 5	675.37	1520.91^{ab}
T5	40.14	546.40	1570.11^{ab}	506.26	681.07	1529.97^{ab}
T6	38.10	553.15	1586.91^{abc}	513.05	695.50	1546.81^{abc}
T7	39.05	555.43	1599.73^{abc}	515.37	706.91	1559.68^{abc}
T8	41.19	557.01	1631.69^{bc}	516.82	717.53	1591.50^{bc}
T9	40.17	562.05	1646.16^{c}	521.88	724.32	1605.99^{c}
P-value	0.230	0.130	0.031	0.166	0.051	0.033

Means with different superscript within column differ significantly (P<0.05).

Table 3: Feed intake and feed conversion ratio (FCR) in broilers fed on different dietary treatments.

Treatment	FCR			Feed Intake (g)		
	0-3 Week	4-6 Week	0-6 Week	0-3 Week	4-6 Week	0-6 Week
T1	1.65	3.23	1.98	813.73	2182.44	2946.17
T2	1.61	2.97	1.87	831.42	2133.75	2965.17
T3	1.62	3.09	1.92	816.24	2132.63	2948.86
T4	1.68	3.19	1.97	838.02	2145.94	2983.96
T5	1.63	3.17	1.95	824.72	2151.21	2975.93
T6	1.59	3.12	1.93	817.77	2169.46	2987.23
T7	1.60	3.06	1.91	822.46	2157.32	2979.79
T8	1.60	3.02	1.88	826.84	2163.77	2990.61
T9	1.56	2.97	1.85	814.30	2148.09	2982.40
P-value	0.609	0.354	0.452	0.987	0.996	1.00

Table 4: Mean log10 value of Lactobacillus spp. Salmonella spp. and *E.coli* isolated from broiler chicken intestine.

Treatments	Lactobacillus spp			Salmonella spp			*E.coli*		
	Crop	Ilium	Caeca	Crop	Ilium	Caeca	Crop	Ilium	Caeca
T1	8.38^{a}	8.61^{a}	8.40^{a}	7.31^{c}	7.14^{c}	7.16^{b}	7.35^{b}	7.17^{c}	7.23^{c}
T2	8.58^{b}	8.69^{ab}	8.35^{a}	6.94^{ab}	6.99^{ab}	6.92^{a}	7.19^{a}	7.00^{a}	6.98^{a}
T3	8.84^{d}	8.87	8.71^{b}	6.92^{a}	7.07^{abc}	7.13^{b}	7.26^{ab}	7.02^{ab}	7.10^{abc}
T4	8.69^{c}	8.75^{bc}	8.61^{b}	7.28^{c}	7.11^{bc}	7.12^{b}	7.33^{b}	7.14^{bc}	7.21^{bc}
T5	8.81^{d}	8.77^{bcd}	8.65^{b}	7.25^{c}	7.10^{bc}	7.10^{b}	7.31^{b}	7.13^{abc}	7.17^{bc}
T6	8.84^{d}	8.80^{cd}	8.70^{b}	7.23^{c}	7.06^{abc}	7.07^{b}	7.25^{ab}	7.13^{abc}	7.14^{bc}
T7	8.75^{cd}	8.86^{d}	8.64^{b}	7.26^{c}	7.05^{abc}	6.95^{a}	7.31^{b}	7.12^{abc}	7.12^{abc}
T8	8.83^{d}	8.97^{e}	8.70^{b}	7.03^{b}	7.02^{ab}	6.94^{a}	7.27^{ab}	7.10^{abc}	7.10^{abc}
T9	9.03^{e}	9.03^{e}	8.93^{c}	6.91^{a}	6.95^{a}	6.92^{a}	7.20^{a}	7.03^{ab}	7.07^{ab}
P-value	0.000	0.000	0.000	0.000	0.024	0.00	0.014	0.052	0.023

Means with different superscript within column differ significantly (P<0.05).

Table 5. Humoral and Cell mediated immune response of broilers under different dietary treatments

Treatments	HA Titre	CMI (mm)
T1	$6.25^{a}\pm0.40$	0.46 ± 0.07^{a}
T2	$8.38^{cd}\pm0.37$	0.52 ± 0.04^{a}
T3	$8.00^{bcd}\pm0.67$	0.51 ± 0.05^{a}
T4	$6.38^{a}\pm0.37$	0.46 ± 0.03^{a}
T5	$6.63^{ab}\pm0.44$	0.51 ± 0.05^{a}
T6	$7.75^{bc}\pm0.55$	0.56 ± 0.04^{ab}
T7	$8.00^{bcd}\pm0.41$	0.59 ± 0.04^{ab}
T8	$8.75^{cd}\pm0.32$	0.72 ± 0.10^{b}
T9	$9.25^{d}\pm0.43$	0.75 ± 0.10^{b}
P-Value	0.000	0.012

Means with different superscript within column differ significantly (P<0.05).

Figures

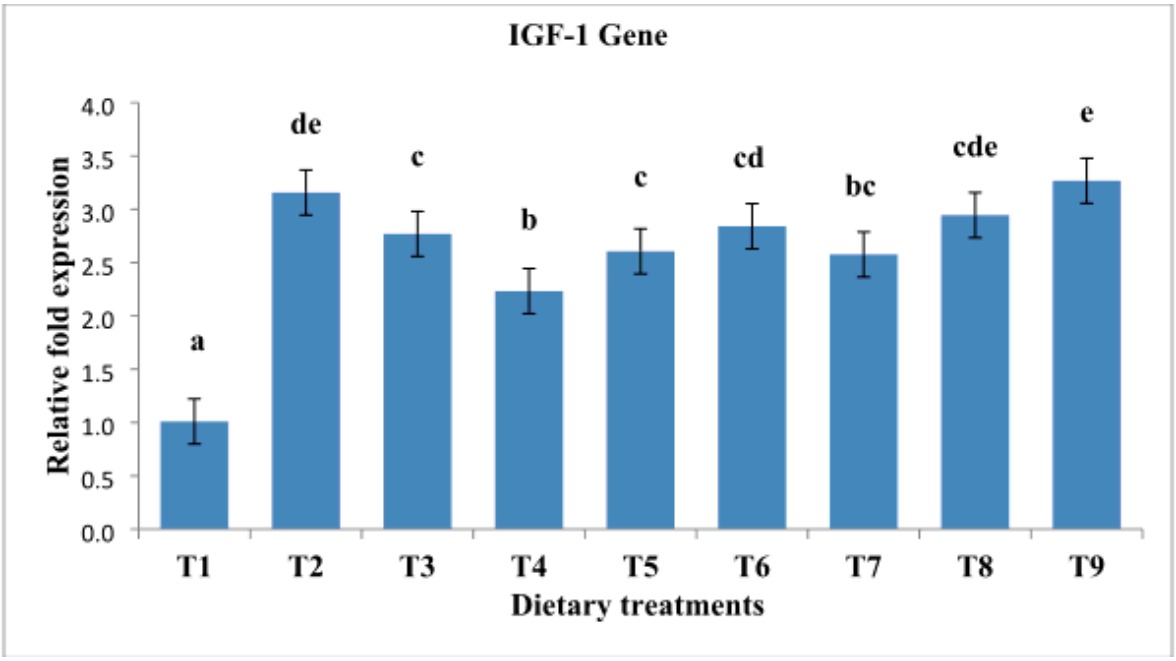

Figure 1

Relative fold expression of IGF-1 gene in muscle tissue of broiler chicken at 6 wk of age under different dietary treatments. Different superscripts indicates significant expression at the level of 95% ($p \leq 0.05$)

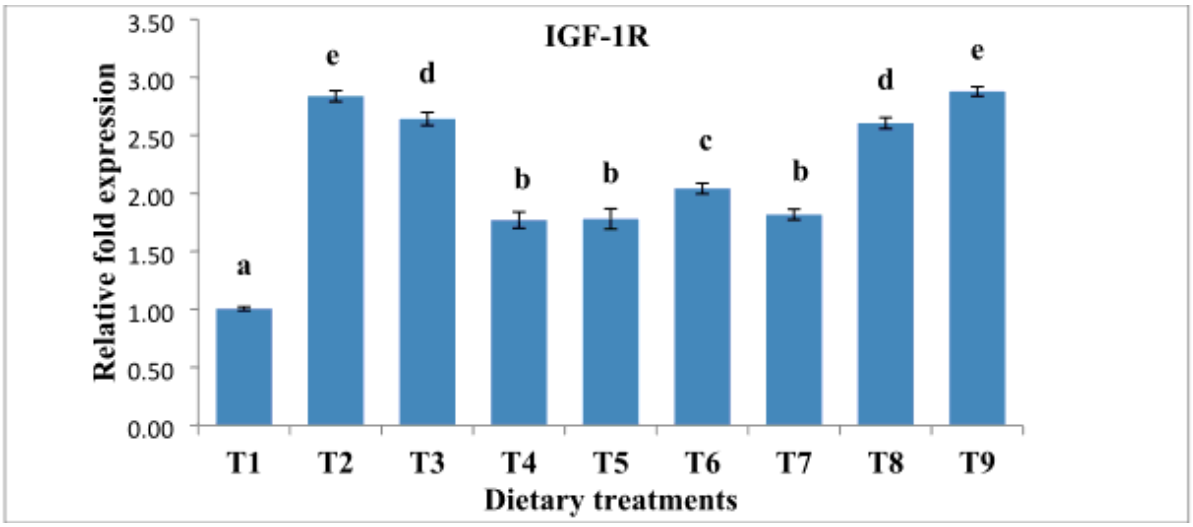

Figure 2

Relative fold expression of IGF-1R gene in Muscle tissue of Broiler chicken at 6 wks of age under different dietary treatments. Different superscripts indicates significant expression at the level of 95% (p≤ 0.05)

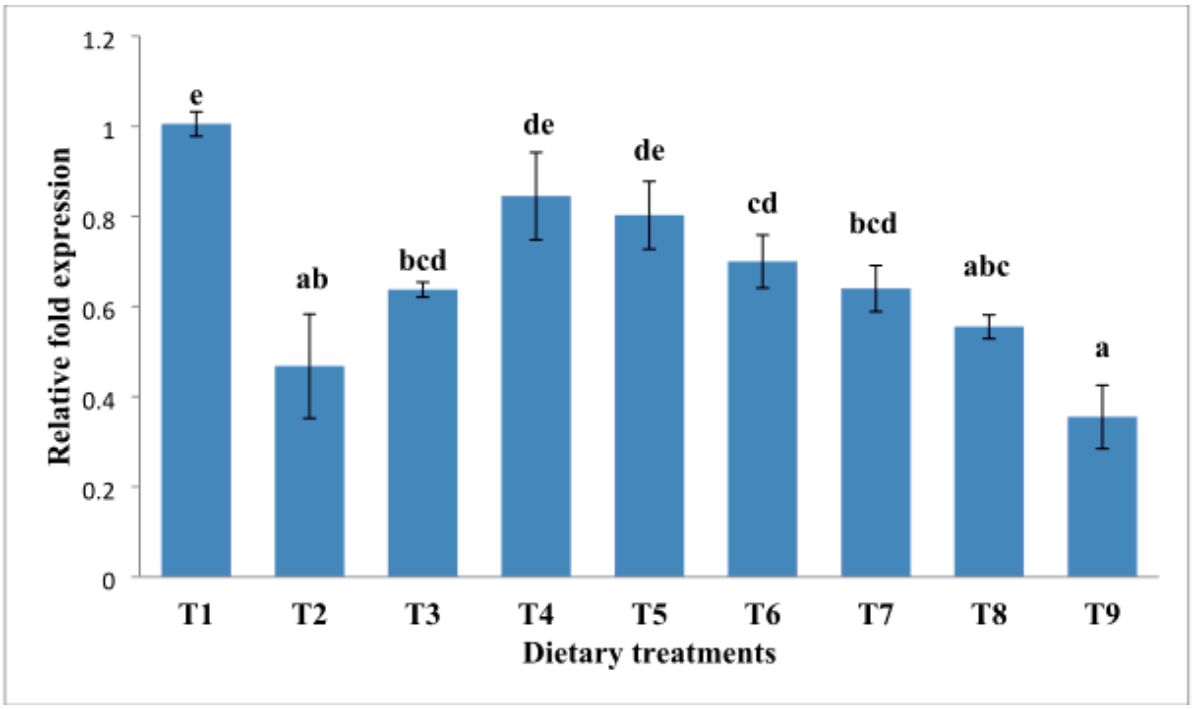

Figure 3

Relative fold expression of IL-6 gene in Ileum tissue of broiler chicken at 6 wks of age under different dietary treatments. Different superscripts indicates significant expression at the level of 95% (p≤ 0.05)

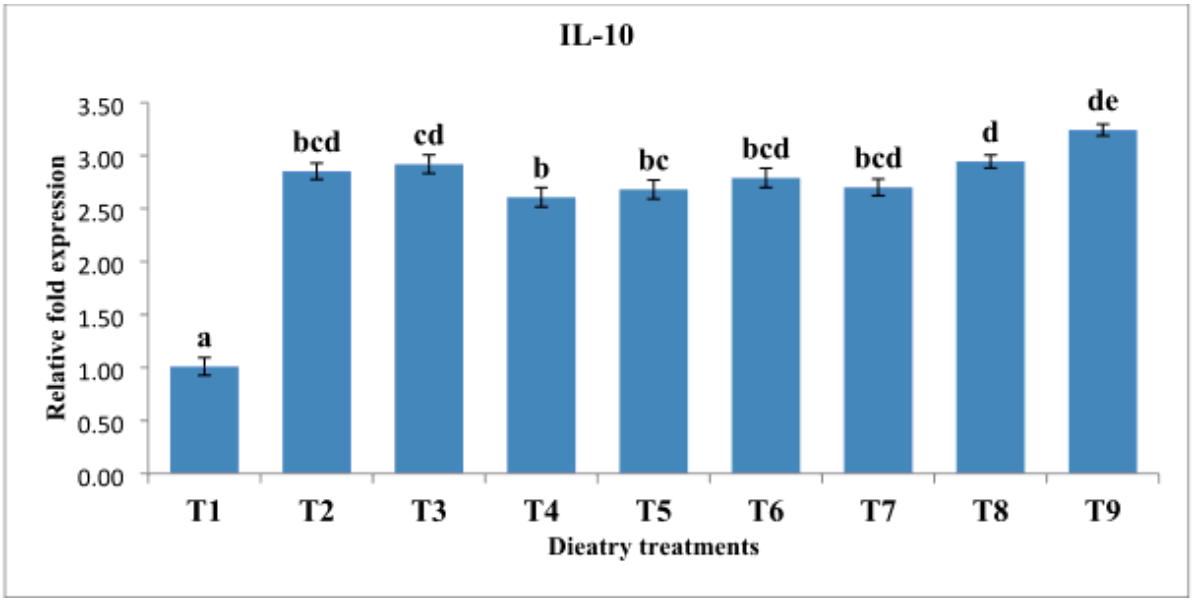

Figure 4

Relative fold expression of IL-10 gene in Ileum tissue of Broiler chicken at 6 wks of age under different dietary treatments. Different superscripts indicates significant expression at the level of 95% (p≤ 0.05)

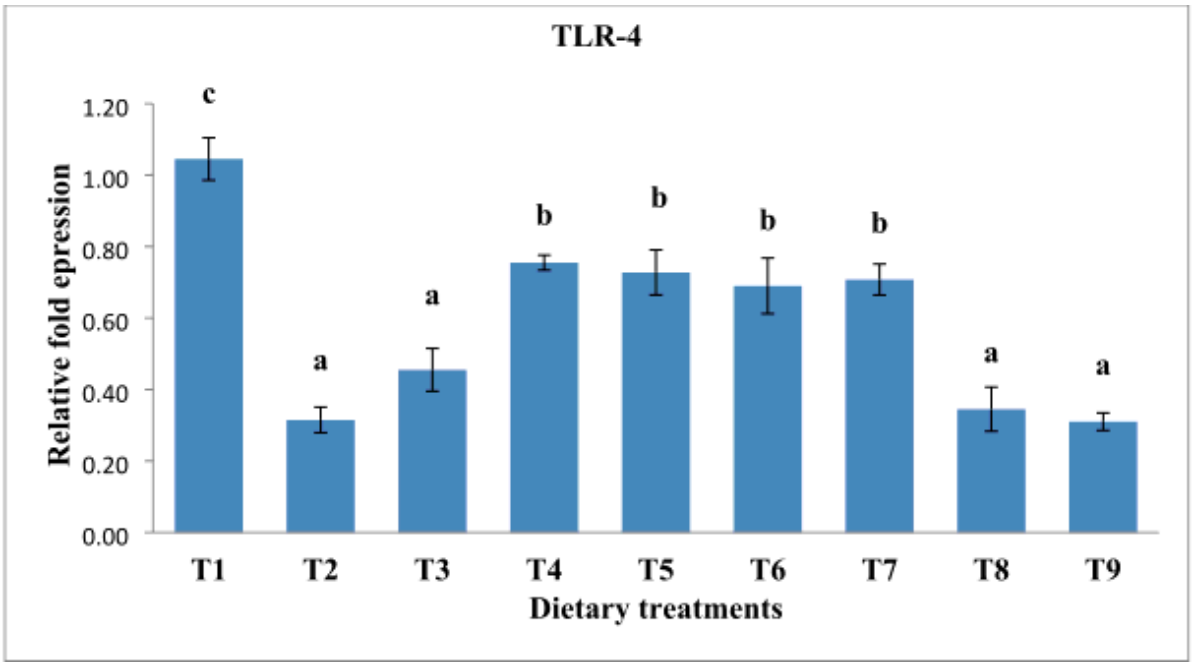

Figure 5

Relative fold expression of TLR-4 gene in Ileum tissue of Broiler chicken at 6 wks of age under different dietary treatments. Means with different superscripts differ significantly (p≤ 0.05)